Original Research

Escherichia coli lipopolysaccharide impairs the calcium signaling pathway in mesangial cells: role of angiotensin II receptors

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Abstract

Sepsis causes impaired vascular reactivity, hypotension and acute renal failure. The ability of the *Escherichia coli* endotoxin (lipopolysaccharide [LPS]) to impair agonist-induced contractility in mesangial cells, which contributes to LPS-induced renal dysfunction, was evaluated. Agonist-induced intracellular calcium ($[Ca^{2+}]i$) mobilization was analyzed using angiotensin II (AngII). The effect of LPS on the levels of the renin–angiotensin system (RAS) components and the roles of vasodilatation-inducing molecules including AT2 receptor (AT2R) and nitric oxide (NO) in the cell reactivity were also evaluated. Confluent human mesangial cells (HMCs) were stimulated with LPS (0111-B4, 100 μg/mL). AngII-induced $[Ca^{2+}]i$ mobilization was measured by fluorometric analysis using Fura-2AM in the absence and presence of an AT2R antagonist (PD123319). The mRNA and protein levels for angiotensinogen, renin, angiotensin-converting enzyme, AT1R and AT2R were analyzed by realtime reverse transcriptase-polymerase chain reaction and Western blot, respectively. NO production was measured by the chemiluminescence method in the culture media after 24, 48 and 72 h of LPS incubation. After 24 h, LPS-stimulated HMCs displayed lower basal $[Ca^{2+}]i$ and an impaired response to AngII-induced rise in $[Ca^{2+}]i$. LPS significantly increased AT2R levels, but did not cause significant alterations of RAS components. PD123319 restored both basal and AngII-induced $[Ca^{2+}]i$ peak, suggesting an involvement of AT2R in these responses. The expected increase in NO production was significant only after 72 h of LPS incubation and it was unaffected by PD123319. Results showed that LPS reduced the reactivity of HMCs to AngII and suggest that the vasodilatation induced by AT2R is a potential mediator of this response through a pathway independent of NO.

Keywords: sepsis, endotoxin, mesangial cell, intracellular calcium, acute renal failure, AT2 receptor, nitric oxide

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Introduction

Sepsis and septic shock are characterized by systemic vasodilatation, arterial hypotension, acute renal failure (ARF) and high mortality rates. The underlying mechanisms responsible for the ARF are complex and not yet clearly understood. Many inflammatory mediators and endocrine mechanisms are involved in the pathogenesis of ARF in sepsis. A variety of vasodilator substances, in particular nitric oxide (NO), are released to compensate for the upregulation of vasoconstrictors,[1] and cause a decrease in systemic vascular resistance and impaired vascular reactivity.[2–4] Paradoxically, in contrast to the systemic vasodilatation, sepsis causes intrarenal vasoconstriction, reduction of the renal plasma flow and glomerular filtration, which leads to ARF. We have previously observed in endotoxemic rats that the decreases in both total and single-nephron glomerular filtration rates were due to decreases in renal plasma flow resulting from increased renal vascular resistance.[5] The glomerular ultrafiltration coefficient (K_f) was not changed in this model, although a 50% decrease in K_f was found in endotoxemic pregnant female rats.[6] Thus, the contribution of K_f to the decrease in glomerular filtration rate during sepsis is still not clearly established. K_f is determined by the glomerular hydraulic permeability coefficient (k) and by the glomerular surface area (S). The latter is in turn directly influenced by the mesangial cells (MCs) due to their contraction/relaxation properties in response to vasoactive substances such as angiotensin II (AngII). Moreover, MCs are known to locally produce AngII[7,8] that may cause MC inflammation and contraction, which is

mediated by an increase in intracellular calcium concentration ([Ca^{2+}]i). Although sepsis is characterized by hyperactivation of the circulating renin–angiotensin system (RAS), little is known about how MCs react to AngII during sepsis. It was recently demonstrated that *Escherichia coli* lipopolysaccharide (LPS) suppressed the RAS in MCs due to a reduction in renin activity.[9] In order to better understand the role of MCs in LPS-induced ARF, the present study evaluated the effects of LPS on MC function. We evaluated the effect of *E. coli* LPS on the ability of cultured MCs to react to AngII with modifications in [Ca^{2+}]i. The role of vasodilator factors such as NO and AngII type 2 receptor (AT2R) activation was also evaluated. Results showed that LPS-stimulated MCs under-reacted to AngII stimulus, with a lower increase in [Ca^{2+}]i. Activation of AT2R was the main mechanism involved in this response.

Methods

MC culture

Experiments were performed with immortalized human mesangial cells (HMCs), kindly provided by Dr Bernhard Banas (Munich, Germany). Cells between passages 60 and 65 were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 5 mmol/L glucose and supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a humidified incubator at 37°C and 5% CO_2. After reaching confluence, cells were washed with phosphate-buffered saline (PBS) and kept for 24 h in DMEM 0.5% FBS. *E. coli* LPS (serotype 0111-B4; Sigma Co., St Louis, MO, USA) in a concentration of 100 μg/mL or vehicle (PBS buffer) were added to the culture medium. Cells were stimulated with LPS up to 72 h. An additional group of cells was simultaneously treated with LPS or an AT2R antagonist, PD123319 (Sigma Co.).

[Ca^{2+}]i measurements

Confluent MCs were resuspended at a concentration of approximately 10^6 cells/mL in 2.5 mL Tyrode buffer (137 mmol/L NaCl, 2.68 mmol/L KCl, 1.36 mmol/L $CaCl_2$, 0.49 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.36 mmol/L NaH_2PO_4 and 5.5 mmol/L D-glucose) containing 0.2% bovine serum albumin and stored in a CO_2 incubator at 37°C for 30 min. The cell suspension was then centrifuged at 1500 rpm for 4 min. Then the supernatant was aspirated, and the pellet was resuspended in 2.5 mL albumin-free Tyrode and transferred to the quartz cuvette for auto-fluorescence determination in a spectrofluorimeter (Photon Technology International, Ontario, Canada). Measurements were made at 340 and 380 nm excitation wavelengths, with emission at 505 nm. The auto-fluorescence ratio was less than 10%. HMCs were then incubated with 0.01% Pluronic-127 detergent and 2 μmol/L of the fluorescent marker Fura-2AM, and the cuvette was transferred to a spectrofluorimeter to record the Fura-2AM fluorescence spectrum in the excitation range of 300–400 nm, with emission at 520 nm. In the etherified form, maximum fluorescence of Fura-2AM was observed at 390 nm. Within one hour, the indicator was transformed to its acidic form, Fura-2, and the fluorescence peak shifted to 350 nm, thus indicating the maximum amount of indicator was incorporated into the cell suspension. Cells were washed with 15 mL Tyrode and centrifuged at 1500 rpm for four minutes. The supernatant was discarded and the pellet was resuspended in 2.5 mL Tyrode and transferred to a spectrofluorimeter programmed for excitation at two wavelengths (340 and 380 nm) with emission at 505 nm, with constant stirring at 37°C. The first reading of this phase corresponded to basal [Ca^{2+}]i levels. The cells were then stimulated with AngII (10^{-6} mmol/L). At the end of each experiment, 50 μmol/L digitonin, 1 mmol/L manganese chloride and 2 mmol/L ethylene gylcol tetraacetic acid were added to the cells. The results are reported as the relative ratio of the 340 and 380 nm wavelengths, using the reading for digitonin to be 100%. The calcium concentration was estimated by the formula of Grynkiewicz *et al.*[10]

NO production

NO production was determined in the culture medium by the chemiluminescence method[11] using the Nitric Oxide Analyzer (NOA™, Model 280; Sievers Instruments, Boulder, CO, USA). NO concentration was normalized by total cellular protein content, which was measured by the method of Lowry.

mRNA expression levels by realtime PCR

Total RNA was purified from HMCs by the phenol and guanidine isothiocyanate-cesium chloride method using an appropriate kit (Trizol Gibco BRL, Rockland, MD, USA). Total RNA (2 μg) was treated with DNase (RQ1 RNase-free DNase; Promega, Madison, WI, USA) to avoid genomic DNA contamination. The RNA pellet was resuspended in RNase-free water and reverse transcribed into cDNA by the addition of a mixture containing 0.5 mg/mL of oligo d(T), 10 mmol/L of DTT, 0.5 mmol/L of deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Uppsala, Sweden) and 200 U of reverse transcriptase enzyme (SuperScript RT; Gibco BRL). The mRNA expression levels for the AT1 and AT2 receptors (AT1R and AT2R), angiotensinogen (Angt), renin, angiotensin-converting enzyme (ACE), sodium calcium exchanger (NCX) and β-actin were estimated by real-time reverse transcriptase-polymerase chain reaction using the GeneAmp 5700 and ABI Prism 7700 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). The primers for PCR amplification were designed using the Primer Express™ software (Applied Biosystems) and chosen based on their efficiency. The following primers were used (forward and reverse, respectively): Angt (attcctgtttgctgtgtatga and aggcttctactgctcactcc), renin (ccaccttcatccgaaagttc and gtctccatctgagggcataag), ACE (ccagttttacccgaaatacg and caccaccaagtcatagatgttg), AT1R (acagctatggaataccgctggc and gctgagacacgtgagtagaaaca), AT2R (gtctcaaagaagaaatccctggc and ctgacgtctcgaaaataaaatgttg), NCX (acatctgcactgtctggcac and ttaaaggcccgttggtcacc) and β-actin (gtacgttgctatccaggctgtg and tacccctcgtagatgggcac). One microliter of each cDNA mixture was added to the PCR. Realtime PCR product

accumulation was monitored using the intercalating dye SYBR Green I (Molecular Probes, USA), which exhibits a higher fluorescence upon binding to double-stranded DNA. The reactions were cycled under conditions previously determined by conventional PCR. Fluorescence for each cycle was quantitatively analyzed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). At the end of the PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min. Fluorescence was measured every 15 s to construct the melting curve used to verify the absence of unspecific amplification products. Relative gene expression was calculated using early PCR stage conditions, under which the amplification curve is logarithmic. For each sample, the amplification curve was crossed by a threshold line, and the PCR cycle needed to achieve this threshold was called 'Ct'. Smaller Ct numbers were correlated with larger numbers of initial copies. Results were normalized by subtracting the Ct obtained for the housekeeping gene β-actin from the Ct obtained for each molecule, producing a ΔCt. As it is uncommon to use ΔCt as a relative expression due to its logarithmic characteristic, the $2^{-\Delta Ct}$ parameter was used to express the relative expression data, which were analyzed as mean $\pm$ SE.

Determination of protein expression levels by Western blot

HMCs were lysed with 600 μL of lysis buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2.5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethanesulfonylfluoride and 44 mmol/L *o*-phenanthroline) per plate (100 $\times$ 20 mm). The lysates were centrifuged at 12,000*g* for five minutes at 4°C and the supernatants were stored at -80°C until use. Protein concentrations were determined with the reagents from BioRad using a calibration curve containing bovine serum albumin. A hundred microgram quantities were separated by 7.5–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli[12] and electrophoretically transferred to nitrocellulose membranes using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Non-specific binding sites were blocked with 5% non-fat dry milk in TBS buffer containing 10 mmol/L Tris-HCl pH 7.5, 200 mmol/L NaCl. Immunoblots were incubated overnight at 4°C with the following primary antibodies: AT1 receptor (1:200 dilution, AT1R; Santa Cruz Biotechnology, Santa Cruz, CA, USA); AT2 receptor (1:200 dilution AT2R; Santa Cruz Biotechnology); and β-actin (1:400 dilution; Abcam, Cambridge, MA, USA). After the membranes were washed three times with TBS-T (Tris-HCl pH 7.5, 200 mmol/L NaCl, 0.05% Tween-20), they were incubated for one hour at 4°C with peroxidase-conjugated secondary antibodies (1:30,000 dilution; Sigma) and then washed three times with TBS-T. Specific protein bands were visualized using the ECL detecting reagents (GE Healthcare, Buckinghamshire, UK) and recorded on Amersham Hyperfilm ECL (GE Healthcare). Optimally exposed autoradiographs were digitally scanned and analyzed using an Imaging Densitometer (Model GS670, Bio-Rad, Hercules, CA, USA) and a Quantity One-GS710 software.

Flow cytometry

Apoptosis and necrosis were determined using flow cytometry by labeling cells with annexin V-FITC and propidium iodide (PI). Adherent cells were trypsinized and resuspended in 200 μL of binding buffer (10 mmol/L HEPES, pH 7.5, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). Subsequently, 4 μL of annexin V-FITC conjugate and 4 μL of PI were added and allowed to bind for 10 min at room temperature in the dark. Detection of viable cells (annexin V-FITC-negative, PI-negative), early apoptotic cells (annexin V-FITC-positive, PI-negative), apoptotic-lysed cells (annexin V-FITC-positive, PI-positive) and necrotic cells (annexin V-FITC-negative, PI-positive) was performed using a Becton Dickinson FACScan instrument in combination with Cell Quest software (Becton Dickinson, Heidelberg, Germany). A total of 10,000 events were used for analysis.

Statistical analysis

Results are presented as mean $\pm$ standard error. The effects of LPS were evaluated using the unpaired Student's *t*-test. Experiments using the AT2R blocker were analyzed using one-way analysis of variance followed by Dunn or Tukey tests when appropriate. The level of statistical significance was defined as $P < 0.05$.

Results

There was no difference between groups in terms of cell viability and the presence of apoptosis or necrosis. LPS treatment did not change the mRNA expression levels for angiotensinogen, renin or ACE and it caused a slight but significant ($P < 0.05$) reduction in AT1R. In contrast, LPS induced a three-fold increase in AT2R mRNA levels (Figure 1a). Similar alterations in AT1R and AT2R protein levels were also observed (Figures 1b and c).

The functional significance of these protein and mRNA changes of the AngII receptors was evaluated by examining the changes in $[Ca^{2+}]i$ in response to AngII stimulus. The results presented in Figure 2 show that the unstimulated mean basal $[Ca^{2+}]i$ was significantly lower in cells exposed to LPS than in control cells. As expected, AngII stimulation increased the $[Ca^{2+}]i$ in both control (2.5-fold) and LPS-treated cells (4-fold). Although the incremental change was higher in LPS cells, the final $[Ca^{2+}]i$ was still significantly lower in the LPS group as compared with the control cells (192 ± 27 versus 358 ± 36 nmol/L, $P < 0.05$). Considering the significant change in the expression of AT2R, we evaluated the effect of an AT2R antagonist (PD123319) on cell sensitivity to AngII stimulation. Simultaneous treatment with LPS and PD123319 restored both the basal $[Ca^{2+}]i$ and the response to AngII to control levels. Also, PD123319 induced normalization in AT1R and AT2R protein expressions (Figure 3).

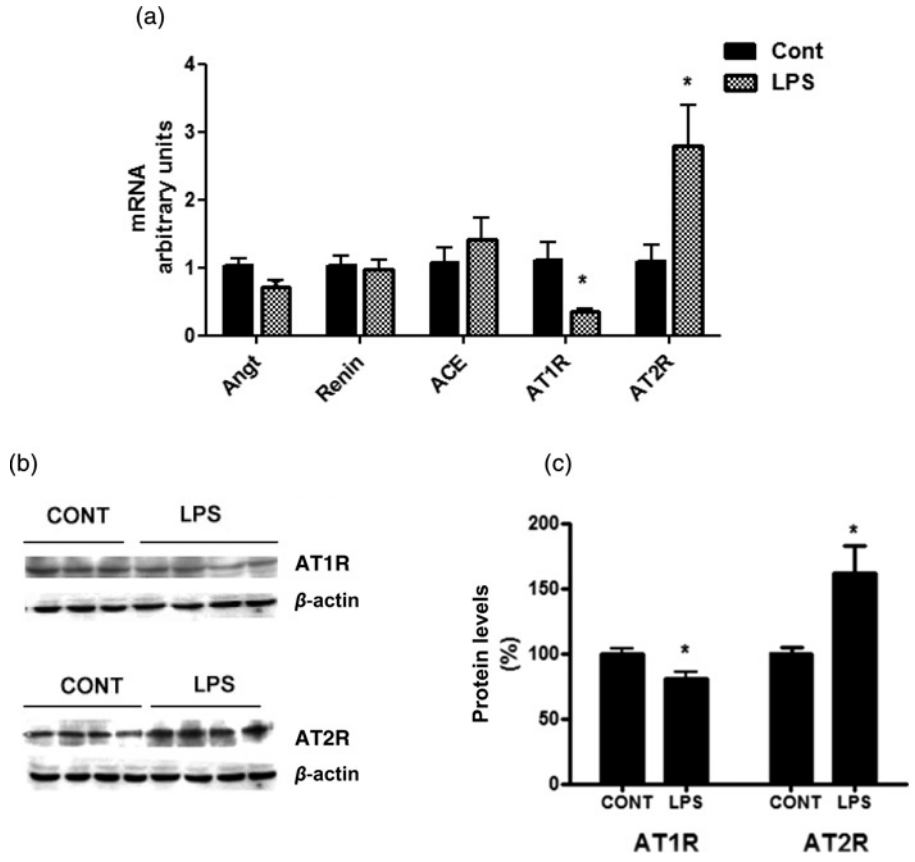

Figure 1 Expression levels for RAS components in control (CONT) and LPS-stimulated cells. (a) mRNA expression levels ($n = 5$). (b) Representative Western blots for AT1R and AT2R. Protein levels were estimated by densitometry analysis of the bands normalized by β-actin levels and expressed as a percentage of the control samples, shown in panel c. $^{*}P < 0.05$ versus control. LPS, lipopolysaccharide; ACE, angiotensin-converting enzyme; Angt, angiotensinogen; AT1R, AT1 receptor: AT2R, AT2 receptor

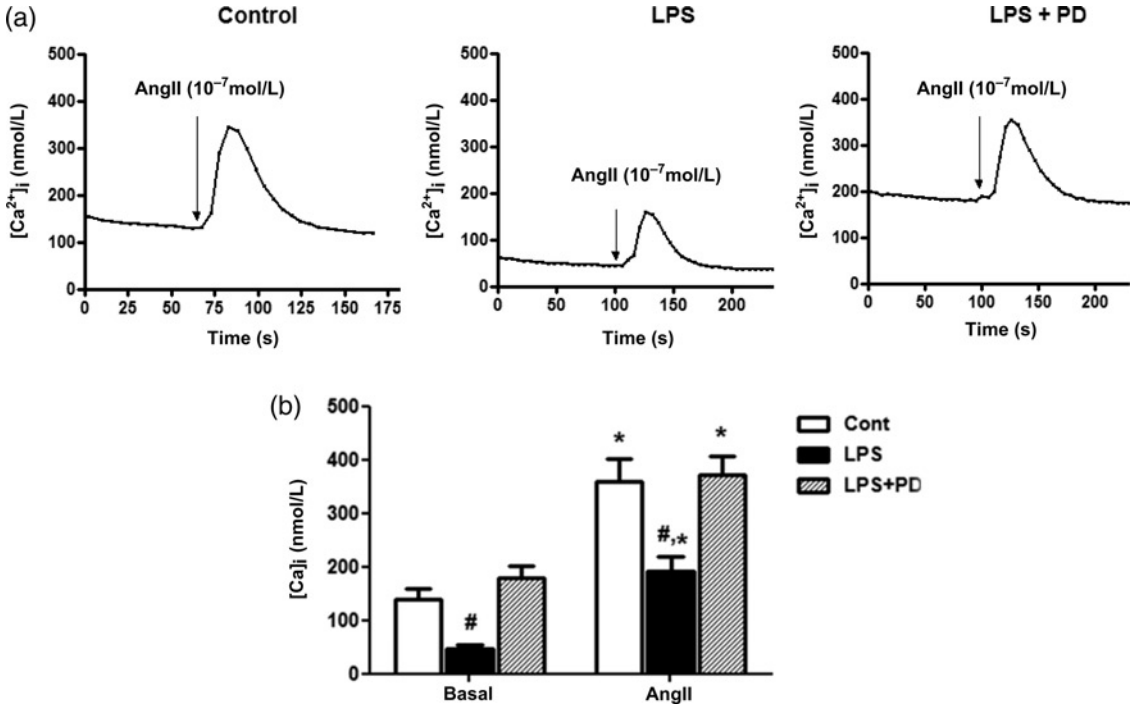

Figure 2 Intracellular calcium concentration. (a) Sample traces showing the peak $[Ca^{2+}]i$ mobilization induced by AngII in control cells and in cells stimulated with LPS for 24 h in the absence or presence of AT2R antagonist (PD123319). (b) Graphic representation of all samples ($n = 5$ for each group), $^{*}P < 0.05$: versus basal; $^{\#}$versus control

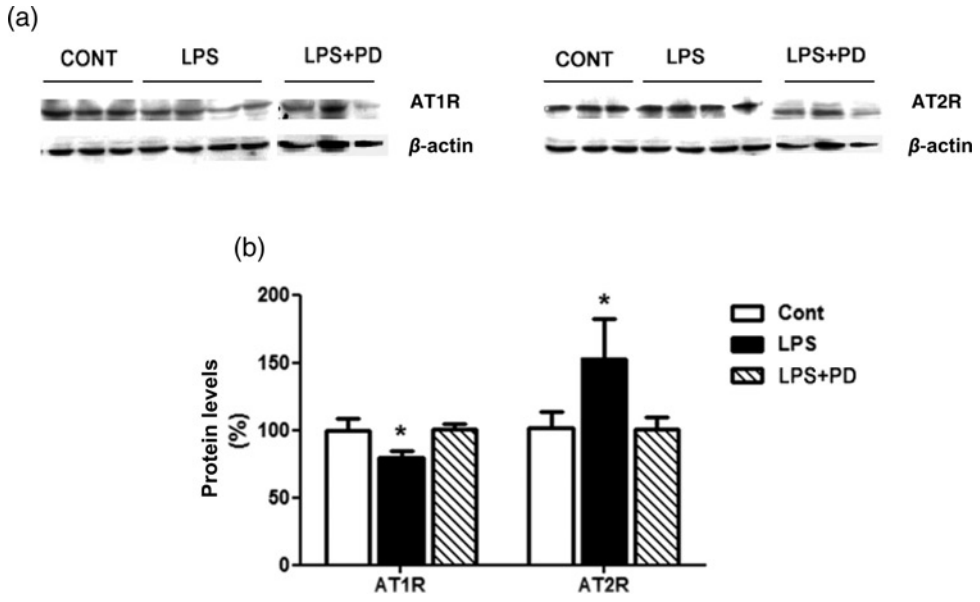

Figure 3 Effect of PD123319 on expression levels of AT1R and AT2R proteins. (a) Representative Western blot showing control cells and LPS-stimulated cells in the absence or presence of PD123319. (b) Protein levels were estimated by densitometry analysis of the bands normalized by β-actin levels and expressed as percentage of the control samples, as shown in panel b. $^{*}P < 0.045$ versus control

Basal $[Ca^{2+}]i$ homeostasis is strongly dependent on the Na^{+}/Ca^{2+} exchanger (NCX) in MCs. In order to determine the involvement of the NCX in the reduction of basal $[Ca^{2+}]i$, we evaluated the mRNA expression levels of this transporter. There was an insignificant decrease in NCX mRNA expression in the LPS group, and PD123319 treatment had no effect on NCX mRNA levels (data not shown). This result suggests that the mechanism of reduction in basal $[Ca^{2+}]$ is not dependent of the expression level of this exchanger.

As expected, LPS stimulation increased NO synthesis in MCs, which was higher after 72 h of incubation (Figure 4). Next, we evaluated the association between NO and AT2R by treating MCs simultaneously with LPS and PD123319. As shown in Figure 4, NO synthesis was not modified in the presence of the AT2R antagonist, suggesting that NO production was not mediated by AT2R in this *in vitro* model.

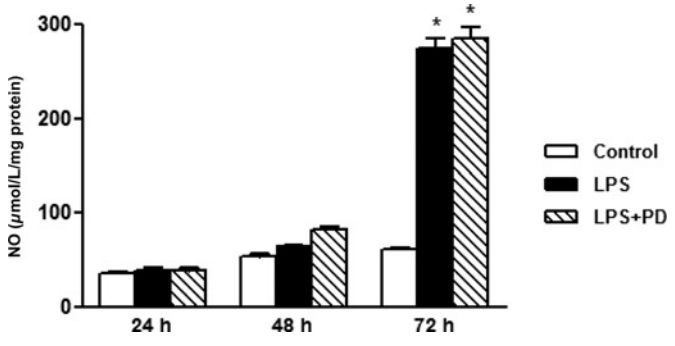

Figure 4 Nitric oxide content (nmol/mg protein) in the culture medium. Time–effect of LPS stimulation in the absence or presence of AT2R antagonist (PD123319)

Discussion

The hemodynamic hallmark of septic shock is systemic vasodilatation, hypotension and reduced vasopressor sensitivity. The decreased renal perfusion pressure results in decreased tissue perfusion, contributing to the development of ARF. Several vasodilator agents have been implicated in the vascular hypocontractility induced by LPS, including NO,[13,14] prostaglandin[15] and bradykinin.[16] In addition, it has been recognized that AT2R mediates the depressor response to AngII.[17] MCs contract and relax in the presence of vasoactive substances. Under pathological circumstances, changes in MC tonus may have a functional significance that contributed to the reduction of the glomerular filtration rate. In the present study we demonstrated that similar to vascular smooth muscle cells, the MCs under LPS treatment also became hyporesponsive to AngII. Moreover, the impairment of agonist-induced $[Ca^{2+}]i$ mobilization was also associated with a reduction in basal $[Ca^{2+}]i$. NCX is a major protein regulating basal $[Ca^{2+}]i$ in MCs.[18] However, the expression of NCX mRNA was not significantly modified by LPS in MCs. Similar results were found by Murray *et al.*[19] using a higher dose of LPS (1 mg/mL) and bradykinin as the agonist agent in rat MCs. The possibility of LPS to induce post-transcriptional changes and interfere with NCX mRNA stability was not addressed in the present study, but will be considered in future studies. On the other hand, low basal $[Ca^{2+}]i$ was normalized by the AT2R antagonist and the AngII-induced increase in $[Ca^{2+}]i$ was completely restored by PD123319.

AngII targets two major receptor types, specifically AT1R and AT2R. The majority of known AngII-induced signaling, including the increase in $[Ca^{2+}]i$ and vasoconstriction, occurs via the AT1R. In contrast, the actions attributed to AT2R stimulation are antagonistic to those

mediated by the AT1R, and many studies have demonstrated that AT2R mediates a depressor response to AngII.[20,21] In the present study, the upregulation of AT2R together with the downregulation of AT1R induced by LPS may constitute a mechanism involved in the reduced response of MCs to AngII. This phenomenon was completely reversed by the AT2R antagonist PD123319 that also restored the expression levels of both AT1R and AT2R. We have no explanation for this effect of PD123319 on AT1R expression, but given that AngII binds with similar affinity to AT1R and AT2R[22] and AT2R counteracts with AT1R, this suggests that the attenuated Ang II-mediated increase in $[Ca^{2+}]i$ induced by LPS was a consequence of an imbalance between the number of AT1R and AT2R with a lower AT1R/AT2R ratio.

Evidence suggests that AT2R is functionally coupled with the NO-cGMP system in the kidney[23,24] and more recently, Gwathmey *et al.*[25] showed that nuclear AT2R stimulates the production of NO in the kidneys of sheep. Since inducible NO synthase (iNOS) is typically increased by cytokines and LPS in MCs,[26,27] we attempted to establish a relationship between AT2R and NO production and elucidate its role in the LPS-induced lower responsivity of MCs to AngII. In the present *in vitro* model, we found that the increase in NO synthesis was significant only after 72 h, while the up-regulation of AT2R was observed earlier, i.e. after 24 h of LPS exposure. In addition, NO production was unchanged by the addition of the AT2R inhibitor. These data suggest that AT2R induced the relaxation of MCs, as represented by the decrease in $[Ca^{2+}]i$, and this reduction was not mediated by NO. In fact, Murray *et al.*[19] demonstrated the iNOS inhibitor N^G-nitro-L-arginine-methyl ester failed to restore the agonist-induced intracellular calcium mobilization after endotoxin exposure in spite of a significant increase in NO synthesis induced by LPS. Taken together these data suggest that impaired $[Ca^{2+}]i$ mobilization induced by LPS is mediated by AT2R, but is dissociated from NO production in HMCs. These results led us to speculate that other mechanisms independent of AT2R and NO, such as the vasodilator cyclooxygenase product(s) and/or voltage-dependent and Ca^{+}-activated K^{+} channels, act as mediators of AT2R-induced vasorelaxation in smooth muscle of rat aortic rings.[28]

In conclusion, these results demonstrate that LPS impaired the vasoconstrictor-agonist-induced calcium mobilization in HMCs by a mechanism dependent on AngII receptors AT1R and AT2R, but independent of NO generation.

Author contributions: All authors significantly contributed to this study.

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REFERENCES

1 Fernandes D, Assreuy J. Nitric oxide and vascular reactivity in sepsis. *Shock* 2008;**30** (Suppl 1):10–13
2 Yaghi A, Paterson NA, McCormack DG. Vascular reactivity in sepsis: importance of controls and role of nitric oxide. *Am J Respir Crit Care Med* 1995;**151**:706–12
3 Schrier RW, Wang W. Acute renal failure and sepsis. *N Engl J Med* 2004;**351**:159–69
4 Schor N. Acute renal failure and the sepsis syndrome. *Kidney Int* 2002;**61**:764–76
5 Lugon JR, Boim MA, Ramos OL, Ajzen H, Schor N. Renal function and glomerular hemodynamics in male endotoxemic rats. *Kidney Int* 1989;**36**:570–75
6 Boim MA, Draibe SA, Ramos OL, Ajzen H, Ulmann A, Schor N. Glomerular hemodynamics during abortion induced by RU 486 and sepsis in rats. *Braz J Med Biol Res* 1994;**27**:1431–44
7 Singh R, Singh AK, Alavi N, Leehey DJ. Mechanism of increased angiotensin II levels in glomerular mesangial cells cultured in high glucose. *J Am Soc Nephrol* 2003;**14**:873–80
8 Vidotti DB, Casarini DE, Cristovam PC, Leite CA, Schor N, Boim MA. High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. *Am J Physiol Renal Physiol* 2004;**286**:F1039–45
9 Almeida WS, Maciel TT, Di Marco GS, Casarini DE, Campos AH, Schor N. Escherichia coli lipopolysaccharide inhibits renin activity in human mesangial cells. *Kidney Int* 2006;**69**:974–80
10 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;**260**:3440–50
11 Hampl VWC, Archer SL. Determination of nitric oxide by the chemiluminescence reaction with ozone. In: Feelish M, Stamler JS, eds. *Methods in Nitric Oxide Research*. New York: John Wiley & Sons, 1996:310–8
12 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–5
13 Cobb JP, Danner RL. Nitric oxide and septic shock. *JAMA* 1996;**275**:1192–6
14 Umans JG, Wylam ME, Samsel RW, Edwards J, Schumacker PT. Effects of endotoxin in vivo on endothelial and smooth-muscle function in rabbit and rat aorta. *Am Rev Respir Dis* 1993;**148**:1638–45
15 Schildknecht S, Bachschmid M, Baumann A, Ullrich V. COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. *FASEB J* 2004;**18**:757–9
16 McLean PG, Perretti M, Ahluwalia A. Inducible expression of the kinin B1 receptor in the endotoxemic heart: mechanisms of des-Arg9bradykinin-induced coronary vasodilation. *Br J Pharmacol* 1999;**128**:275–82
17 Yayama K, Okamoto H. Angiotensin II-induced vasodilation via type 2 receptor: role of bradykinin and nitric oxide. *Int Immunopharmacol* 2008;**8**:312–8
18 Williams I, Williams C, Siroky B, Bates E, Kovacs G, Peti-Peterdi J, Unlap MT, Bell PD. Regulation of mesangial cell Na^{+}/Ca^{2+} exchanger isoforms. *J Cell Physiol* 2004;**199**:181–93
19 Murray PT, Wylam ME, Umans JG. Endotoxin impairs agonist-induced calcium mobilization in rat mesangial cells. *Am J Respir Crit Care Med* 1997;**156**:1846–54
20 Duke LM, Evans RG, Widdop RE. AT2 receptors contribute to acute blood pressure-lowering and vasodilator effects of AT1 receptor antagonism in conscious normotensive but not hypertensive rats. *Am J Physiol Heart Circ Physiol* 2005;**288**:H2289–97
21 Scheuer DA, Perrone MH. Angiotensin type 2 receptors mediate depressor phase of biphasic pressure response to angiotensin. *Am J Physiol* 1993;**264**:R917–23
22 Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab* 2000;**11**:1–6
23 Carey RM. Update on the role of the AT2 receptor. *Curr Opin Nephrol Hypertens* 2005;**14**:67–71
24 Siragy HM, Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 1997;**100**:264–9

25 Gwathmey TM, Shaltout HA, Pendergrass KD, Pirro NT, Figueroa JP, Rose JC, Diz DI, Chappell MC. Nuclear angiotensin II type 2 (AT2) receptors are functionally linked to nitric oxide production. *Am J Physiol Renal Physiol* 2009;**296**:F1484–93
26 Raij L, Baylis C. Glomerular actions of nitric oxide. *Kidney Int* 1995;**48**:20–32
27 Shultz PJ, Archer SL, Rosenberg ME. Inducible nitric oxide synthase mRNA and activity in glomerular mesangial cells. *Kidney Int* 1994;**46**:683–9
28 Fukada SY, Tirapelli CR, de Godoy MA, de Oliveira AM. Mechanisms underlying the endothelium-independent relaxation induced by angiotensin II in rat aorta. *J Cardiovasc Pharmacol* 2005;**45**:136–43

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